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ERGOVALINE TOXICITY ON CACO-2 CELLS AS ASSESSED BY MTT, ALAMARBLUE, AND DNA ASSAYS

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Summary

The exact mechanisms of fescue toxicity in animals have yet to be established, but it has been associated with an inability to thrive. Ergovaline is the major ergopeptine alkaloid associated with fungal infections of tall fescue. Gastrointestinal (GI) toxicity of ergovaline (10^{-11} to 10^{-4} *M*) was evaluated in Caco-2 cells (mimicking the GI epithelium) beginning on days 1, 8, and 18 of culture. Acute and chronic toxicity was assessed after 24 and 72 h of exposure. Treatment periods were chosen to study undifferentiated, semidifferentiated, and completely differentiated cells. Cell loss and metabolic activity were assessed by thiazolyl blue reduction (3-(4,5-dimethylthiozole-2-yl)-2,5,-biphenyl tetrazolium bromide [MTT], mitochondrial succinate dehyrdogenase activity), alamarBlue assay (cytochrome oxidase activity), and deoxyribonucleic acid (DNA) quantitation. Undifferentiated cells were sensitive to 1×10^{-4} *M* ergovaline after acute exposure (from 52 to 74% of control values depending on assay). After 72 h of exposure to 1×10^{-4} *M* ergovaline, in all three assays, treatment means were reduced to ~10% of the control means. By day 11 in culture, ergovaline toxicity to cells had decreased. With 24 h exposure, an apparent paradoxical increase in MTT was seen at some concentrations. This increase in MTT was also found in fully differentiated cells (day 21), whereas alamarBlue activity decreased. No change in DNA was found until 72 h of exposure, when DNA was reduced ~12% over most concentrations. These findings indicate differentiation state–dependent sensitivity of Caco-2 cells to ergovaline, potential problems of the MTT assay as an indicator of cellular toxicity, and usefulness of alamarBlue assay over DNA assay for toxicity assessment.

Key words: ergovaline; ergopeptine; fescue toxicosis; gastrointestinal cells.

Introduction

Ergovaline is one of a class of compounds (ergopeptine alkaloids) that is produced by endophytes (fungi) that infect forages such as fescue (Fig. 1). The association of ergot poisoning with consumption of infected cereals was made centuries ago and the condition was described as "St. Anthony's fire." The symptoms include the vascular effects of the poison, including numb, cold, or blue limbs, and absence of peripheral arterial pulses (Yater and Cahill, 1936). One of these compounds, ergotamine, has been used clinically in the treatment of migraines. (Physicians' Desk Reference, 1995). Consumption of endophyte-contaminated fescue by livestock can result in a syndrome referred to as "fescue toxicosis." The economic losses to the cattle industry due to contaminated fescue have been estimated to be as great as $609 million annually (Hoveland, 1993). The symptoms include inappetence, failure to thrive, inability to regulate body temperature (heat-stress susceptibility), reproductive and lactational failure, and hoof rot (Williams et al., 1975; Rhodes et al., 1991; Porter and Thompson, 1992; Paterson et al., 1995; Blaney et al., 2000; Sharma et al., 2002). Although these symptoms fail to act as conclusive diagnostic indicators, depressed serum prolactin concentrations are reliably indicative of fescue toxicosis (Paterson et al., 1995). The mechanism(s) of toxicity has(ve) yet to be fully understood. Dopamine, serotonin, and norepinephrine agonists–antagonists have been evaluated for their ability to exacerbate or alleviate ergot alkaloid effects because ergot alkaloids are known to cause contraction of smooth muscles and central nervous system symptoms (review by Berde and Schild, 1978; Boling et al., 1989; Samford-Grigsby et al., 1997; Bennett-Wimbush and Loch, 1998).

Studies of ergot alkaloids have indicated a short half-life of circulating ergot peptide alkaloids, with serum half-lives of ~1 h (Nimmerfall and Ronsethaler, 1976). Plasma ergovaline was found to have a half-life of less than 1 h in both sheep and goats after intravenous administration (Jaussaud et al., 1998; Durix et al., 1999). Although tissue concentrations were not reported, others have assumed the presence of ergot alkaloid biological activity in tissues to be reflective of ergot alkaloid concentrations. Rothlin (1946/1947) reported tissue biological activity from highest to lowest to be liver > kidney > lung > spleen > skeletal muscles. Oral administration of ^{14}C-ergotamine (an ergot peptide alkaloid) to monkeys resulted in ~70% fecal, 7% urinary, and 24% biliary elimination, with a presumed absorption of ~31% (Meszaros et al., 1975). These values reflect radioactivity and not necessarily parent compound. Although some work has been published using ruminant gastric tissues to quantitate GI transport (Hill et al., 2001), research efforts have been hindered by the limited availability and expense of purified ergot alkaloids. It is the goal of this laboratory to study the absorption of ergovaline using an established gastrointestinal

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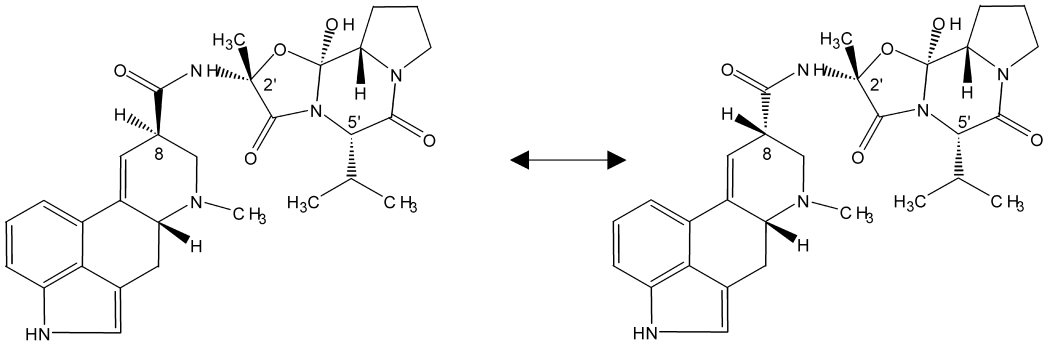

FIG. 1. Structures of ergovaline and ergovalinine, epimerization around C-8.

(GI) cell model, Caco-2 cells derived from a human colon adenocarcinoma. Because GI transport is dependent on the integrity of the monolayer of cells and their tight junctions, first it was necessary to perform toxicity studies with ergovaline. The need for GI toxicity assessment was also implicated in work of Whittemore and Miller (1977), who reported intestinal lesions in growing pigs fed ergotized wheat. The GI tract consists of a continuum of cells—that is, stem cells (rapidly dividing and undifferentiated, located in the crypts), semidifferentiated cells, through fully differentiated cells (incapable of dividing, forming apical microvilli and tight junctions with juxtaposed cells, located in the villi). Therefore toxicity was evaluated in all three states of differentiation.

Three assays were chosen to assess toxicity. Deoxyribonucleic acid (DNA) was chosen because it is commonly accepted as a measure of cell number. In the case of growing cultures, DNA can be used to assess cytostasis when cell division has been decreased relative to controls. Unfortunately, DNA is not a very sensitive indicator of toxicity because the toxic effect will not be registered by changes in DNA until cells have detached from the plate. Therefore, two other assays (3-[4,5-dimethylthiozole-2-yl]-2,5,-biphenyl tetrazolium bromide [MTT] and alamarBlue) were used as indicators of cell toxicity that assess "normal" metabolic activity. The MTT assay has long been used in toxicity studies and is routinely accepted by the scientific community (Denizot and Lang, 1986; Ehrich and Sharova, 2000). The reduction of the chromophore in the MTT assay is dependent on succinate dehydrogenase activity. In the alamarBlue assay (Dayeh et al., 2003), the chromophore replaces molecular oxygen as the electron acceptor at the end of the electron transport chain. It was hoped that by monitoring cell response to ergovaline with three different assays, nontoxic conditions for future transport studies could be established.

MATERIALS AND METHODS

Cell cultures. Caco-2 cells (passage 18) were obtained from American Type Culture Collection (Manassas, VA)[2] and maintained in Falcon flasks (25 cm^2, Becton-Dickinson, Bedford, MA) in Dulbecco modified Eagle medium (DMEM) containing 4.5 g/L glucose and 4 m*M* glutamine and supplemented with 1.7 g/L sodium bicarbonate, 1% nonessential amino acids, 100,000 U/L penicillin, and 100 mg/L streptomycin (all from Sigma Chemical Co., St. Louis, MO, unless otherwise noted). Initially, cultures were maintained in medium containing 20% characterized fetal bovine serum (FBS, HyClone, Logan, UT) but were stepped down to 10% FBS. Humidified incubators were maintained at 37° C and 5% CO_2. Cells were passaged approximately every fourth d (undifferentiated, subconfluent cells) using 0.05% trypsin in phosphate-buffered saline (PBS), pH 7.4, with 4.3 m*M* ethylenediamine-tetraacetic acid for 3 min at 37° C and seeded at 5×10^4 cells/25 cm^2. Cells were fed every other d. Cells for toxicity experiments (passages 50–68) were plated at 1×10^3 cells/well in 96-well plates (Falcon, Becton-Dickinson, Bedford, MA), using media containing 10% FBS. Cells were tested for mycoplasma according to the method of Freshney (1987). The time line for differentiation of cells was found to be consistent with that reported by Briske-Anderson et al. (1997) as assessed by sucrase activity (Messer and Dahlqvist, 1966). For this study, cells on days 0–4 after plating are considered undifferentiated, days 8–11 semidifferentiated, and days 18–21 fully differentiated.

Ergovaline treatment. Ergovaline was synthesized by Forrest Smith, Auburn University, Department of Pharmacal Sciences, Auburn, Alabama, and was 93% pure by high-performance liquid chromatography analysis. Fresh 2×10^{-2} *M* ergovaline stock in methanol was prepared for each experiment. Solvent controls consisted of cells treated with 0.5% methanol (for 1×10^{-4} *M* ergovaline) and 0.05% methanol for all other ergovaline concentrations. Other controls included wells without cells that received medium ± ergovaline. Cells were treated on days 1, 8, and 18 after plating with 1×10^{-4} to 1×10^{-11} *M* ergovaline tartrate. High concentrations required for detection in the absence of radiolabeled compound (for transport studies) were tested to evaluate potential cytotoxicity. After 24 h of exposure, toxicity was assessed on one set of plates, whereas incubations of a second set of plates were continued for a total exposure period of 72 h (Fig. 2, experimental design). The medium (± ergovaline) was replenished on the latter plates after 48 h of exposure. As previously reported (Smith and Shappell, 2002) ergovaline was found to isomerize to 40% ergovalinine (Fig. 1) after 6 h under culture conditions, without further change during the remaining 72 h of incubation. All plates necessary to complete an entire experiment were set simultaneously on day 0. An entire experiment included all plates required to perform all assays (MTT, DNA, and alamarBlue) at all ergovaline concentrations for each stage of differentiation. One experiment was plated using passage 56 cells, with a subsequent experiment from passage 59 cells.

3-(4,5-Dimethylthiozole-2-yl)-2,5,-biphenyl tetrazolium bromide assay. Metabolic activity was determined using a slight modification of the MTT assay described in Current Protocols in Toxicology (2002) and by Strickland et al. (1996). Basically, a stock solution of 2.5 mg/ml MTT or thiazolyl blue was prepared in DMEM without phenol red and filter sterilized (0.22 μm). During the experimental period, stocks were stored in the dark at 4° C (~2 wk). A 20-μl aliquot of MTT was added to the 200 μl of medium on the cells,

[2] Mention of trade names or commercial products in this article is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture.

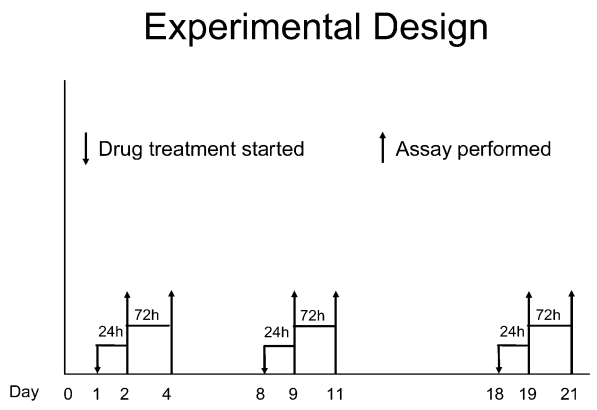

FIG. 2. Experimental design. Day 0 is d of plating.

resulting in 0.23 mg/ml final concentration. Cells were returned to the incubator for 4 h. Supernatant was removed, and cells were solubilized in 200 μl dimethyl sulfoxide (DMSO). Absorbance (A_{590nm}) was measured on a Victor 1420 Multichannel Counter (Wallac, Turku, Finland). Absorbance was found to be linear through 1.5 absorbance units in standardization experiments. The effects of phenol red and FBS in the incubation medium were also evaluated in preliminary experiments. Incubations in the presence of phenol red and FBS gave values that were 95% of those obtained in their absence. Fetal bovine serum and phenol red were left in the MTT-containing incubation medium to avoid nutrient stress and disruption of cells that would be caused by removing FBS and phenol red–containing medium and replacing it with medium without FBS and phenol red.

AlamarBlue assay. Metabolic activity was also assessed with the water-soluble reagent, alamarBlue, essentially as described by Dayeh et al. (2003) and by Trek Diagnostic Systems, Inc., Westlake, Ohio (reagent source). AlamarBlue stock (100×) was diluted with DMEM minus phenol red (GIBCO Invitrogen, Carlsbad, CA) to 11×, filter sterilized (0.22 μm), and stored in the dark at 4° C. After MTT was added to one set of plates, alamarBlue was added to a second set, adding 20 μl of the 11× solution into a well containing 200 μl of medium for a final 1× concentration. Cells were returned to the incubator for 4 h, followed by fluorescence measurement using a Victor 1420 Multichannel Counter (excitation, 545 nm; emission, 590 nm). Bubbles sometimes formed in the wells because of the presence of serum and required popping with a needle before measurement. Linear fluorescence ranges were established in standardization experiments.

Deoxyribonucleic acid assay. The sets of plates used for alamarBlue assays were rinsed with PBS after fluorescence measurement and used for later DNA analysis using the bis-benzidazole (Hoescht 33258) DNA-staining technique of Rago et al. (1990). In brief, cells were rinsed with PBS (pH 7.4), rinsed with 100% ethanol, followed by fixation for 10 min in 100% ethanol, and stored at 4° C in PBS. Later, PBS was removed from cells, and 100 μl of nanopure water was added to each well. Plates were frozen at −20° C for 1 h. After the cell solution thawed, 100 μl of 0.1 *M* sodium phosphate (pH 7.4) 4 *M* sodium chloride buffer (2× phosphate saline buffer, PSB) was added to each well, followed by 20 μl of 110 μg/ml Hoescht in water. A standard curve was prepared in a separate 96-well plate at the same time cells were lysed, using a calf thymus DNA stock (0.1 mg/ml in 1× PSB), ranging from 25 to 8000 ng DNA/well. All wells containing standards were brought to 200 μl using 1× PSB, followed by the addition of 20 μl Hoescht. Solutions in wells were mixed using pipette tips. Plates were held in the dark at room temperature for 1 h, and fluorescence was read on a Victor 1420 multichannel counter (excitation, 355 nm; emission, 460 nm).

Statistical analysis. Data for undifferentiated, semidifferentiated, and differentiated stages of cells were analyzed separately for each exposure time by using a mixed model analysis of variance (SAS/STAT, SAS Institute, Inc., Cary, NC). Treatment was the independent variable, and replicate was used as a blocking factor. The least square means (LSM) of ergovaline treatment values were compared with appropriate solvent controls by using Dunnett's method for multiple comparisons to a control. Statistically significant treatment differences from controls are indicated by the probability values on the graphs.

RESULTS AND DISCUSSION

Cells behaved as previously reported (Briske-Anderson et al., 1997), reaching confluence by approximately day 9, and differentiation increased as reflected by increase in sucrase activity from day 11 to 21 (~14 pmoles to 44 pmoles sucrase/mg protein respectively). All assays for control cells typically indicated the same pattern of increase across time in culture (Table 1). In the presence of high ergovaline concentrations, assay complications arose for both the MTT and the alamarBlue assays. At ergovaline concentrations of 10^{-5} to 10^{-4} *M* for MTT and 10^{-4} *M* for alamarBlue, a visible color change occurred, and alamarBlue fluorescence values of media blanks plus ergovaline (no cells) were found to increase by ~40% (data not shown). In the case of MTT, no change in media blanks plus ergovaline was seen in spectrophotometric readings because supernatant is removed before solubilization with DMSO and measurement of absorbance. Investigators need to include media and treatment blanks in these assays to ensure correction for any potential artifacts. Alternatively, drug treatments could be removed and cells rinsed before the MTT or alamarBlue assay. Two disadvantages of this approach are potential cell recovery if drug has short biological activity half-life and loss of loosely attached cells (especially relevant in late-stage Caco-2 cultures or stressed cultures).

Ergovaline's effect on Caco-2 cells was dependent on concentration, exposure period, and differentiation status of cells (Fig. 3, representative experiment). To display all three assays in one graph, LSM are reported as a percentage of the LSM for the appropriate

TABLE 1

LEAST SQUARE MEANS OF SOLVENT-TREATED CONTROL CELLS[a]

Differentiation state	Harvest day (hour of exposure)	DNA (ng)[b]	AlamarBlue[c] (FU)	MTT[d] (Abs)
Undifferentiated	2 (24)	76/65 (±7.6)	15,362/14,044 (±4002)	0.199/0.183 (±0.0126)
	4 (72)	255/235 (±17.8)	49,788/49,772 (±4463)	0.522/0.512 (±0.0180)
Semidifferentiated	9 (24)	3138/3315 (±212.3)	297,000/307,110 (±2252)	0.951/0.881 (±0.0380)
	11 (72)	3595/3550 (±211.7)	320,890/311,520 (±2680)	0.956/0.886 (±0.0280)
Differentiated	19 (24)	2653/2458 (±155.7)	460,360/419,440 (±1256)	0.813/0.920 (±0.0440)
	21 (72)	3001/2770 (±318.4)	538,560/557,150 (±1846)	0.915/0.981 (±0.037)

[a] Least square means of 0.05% methanol–treated cells/0.5% methanol–treated cells (±pooled SE, n = 6, two replicates).
[b] DNA, deoxyribonucleic acid.
[c] Fluorescence units at excitation λ 545 nm and emission λ 590 nm.
[d] MTT, 3-(4,5-dimethyl thiozole-2-yl)-2,5-biphenyl tetrazolium bromide. Absorbance units at λ 590 nm.

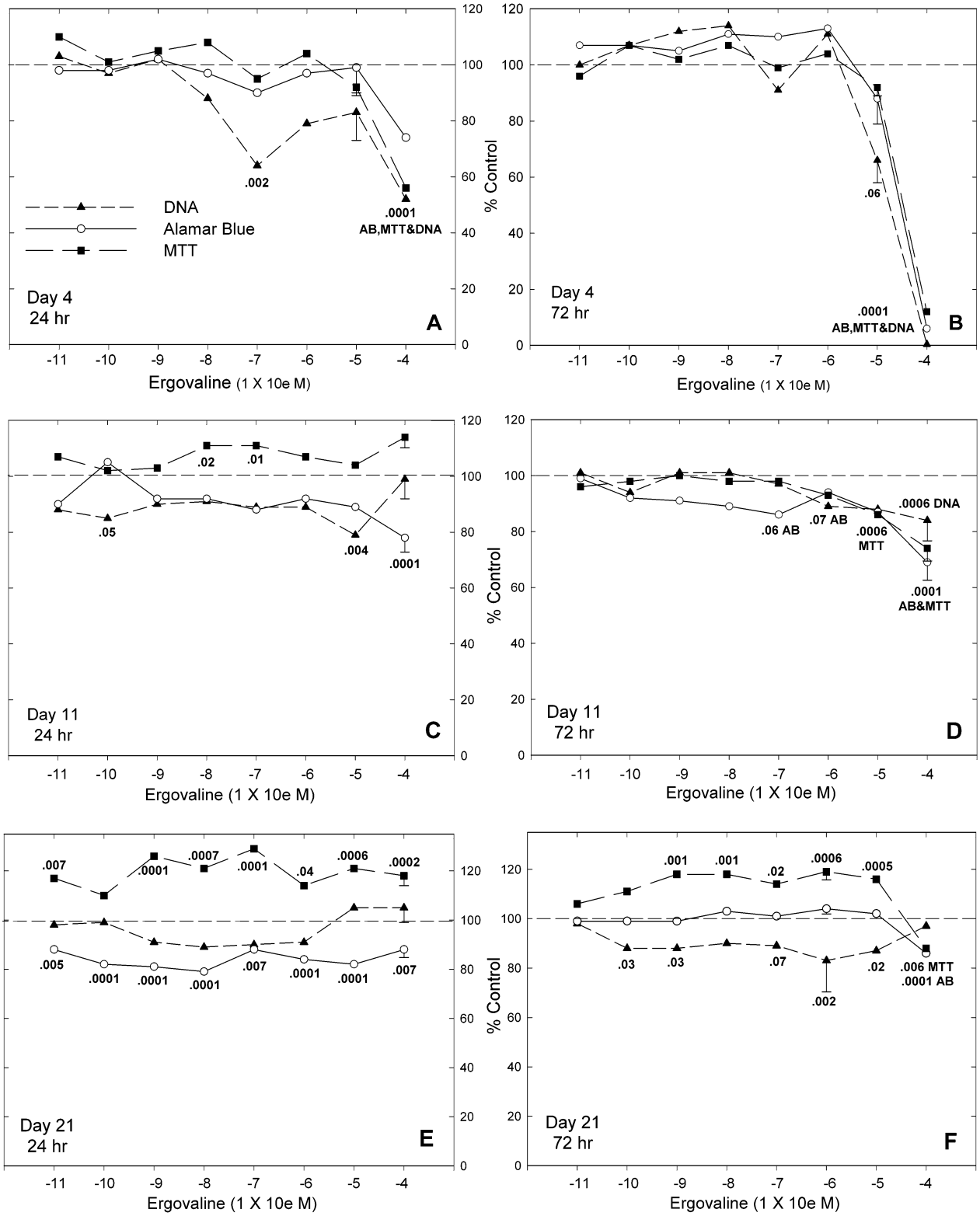

FIG. 3. Effect of ergovaline on Caco-2 cells. Least square means (LSM) of cells treated with ergovaline (time and d indicated on *panel*) for deoxyribonucleic acid (▲), alamarBlue (○), and MTT (■) are represented relative to solvent controls (100%). *Error bars* reflect a representative set of pooled SE (two replicates, n = 6 each). Least square means of ergovaline treatment were statistically different from the solvent control LSM, where indicated by *P* values next to *data points*.

TABLE 2

CELL DENSITY DETERMINATION AFTER 24-H EXPOSURE OF BOVINE DORSAL PEDAL VEIN ENDOTHELIAL CELLS AS A MEASURE OF POTENTIAL CYTOTOXICITY OF TALL FESCUE ALKALOIDS[a,b]

Treatment[c]	Cell density ($OD^{570\ nm}$)—MTT analysis									
	0	$\times10^{-11}$ *M*	$\times10^{-10}$ *M*	$\times10^{-9}$ *M*	$\times10^{-8}$ *M*	$\times10^{-7}$ *M*	$\times10^{-6}$ *M*	$\times10^{-5}$ *M*	$\times10^{-4}$ *M*	SE
α-Ergocryptine	0.485	0.563	0.601	0.497	0.587	0.550	0.525	0.602	0.152[c]	0.028
Ergovaline	0.590	0.522	0.500[d]	0.545	0.561	0.640	0.712	0.465[d]	0.405[d]	0.026
Ergonovine	0.634	0.631	0.574	0.589	0.507[d]	0.512[d]	0.626	0.653	0.559[d]	0.024
Ergine	0.597	0.392[d]	0.495[d]	0.569	0.622	0.560	0.658	0.632	0.520[d]	0.025
Loline	0.635	0.621	0.645	0.638	0.632	0.686	0.577	0.734	0.651	0.025
N-Formyl loline	0.590	0.606	0.616	0.603	0.582	0.625	0.636	0.550	0.539	0.031
N-Acetyl loline	0.507	0.466	0.497	0.571	0.612	0.594	0.560	0.598	0.588	0.027

[a] MTT, 3-(4,5-dimethylthiozole-2-yl)-2,5-biphenyl tetrazolium bromide; SE, standard error; OD, optical density; LSM, least square means.
[b] Strickland et al. (pers. comm.). Endothelial cell cultures established from one tissue sample were treated at confluence.
[c] n = 8; concentration for tall fescue alkaloids; means presented are LSM.
[d] Value is lower ($P < 0.05$) than corresponding treatment.

control. Ergovaline treatment had the greatest effect on undifferentiated cells (*panels A and B*, 24- and 72-h treatment, respectively), and toxicity increased as exposure period increased. Undifferentiated cells are rapidly dividing, so these results reflect the sum effect on both cytostasis and cytotoxicity. After 24 h of exposure, 10^{-4} *M* ergovaline decreased metabolic activity (alamarBlue 26% and MTT 44%) and DNA (48%), $P \leq 0.0001$. When cells were exposed to 10^{-4} *M* for 72 h, toxicity increased and metabolic activity and DNA of treated cells were only ~10% or less of those of control cells ($P \leq 0.0001$). Deoxyribonucleic acid was also decreased after 72 h of treatment with 10^{-5} *M* ergovaline ($P = 0.06$).

As cells differentiated (day 11), ergovaline toxicity was diminished (Fig. 3, *panels C* and *D*). Deoxyribonucleic acid tended to be slightly lower across all except the highest concentrations after 24 h of exposure. AlamarBlue results mirrored the trend seen for decreased DNA with the exception that 10^{-4} *M* resulted in a significant decrease (78% of control, $P \leq 0.0001$). In contrast, MTT values were slightly elevated at some concentrations (111% at 10^{-8} and 10^{-7} *M*; $P = 0.02$ and 0.01, respectively). When the exposure period was extended to 72 h, a significant decrease in DNA and metabolic activity was reflected by all assays, yet treated cell means were reduced by only ~20–30% compared with the ~90% seen for undifferentiated cells (16% for DNA, $P = 0.0006$; 26% for MTT, $P \leq 0.0001$; and 31% for alamarBlue, $P = 0.0006$).

In fully differentiated cultures (day 21), assessment of ergovaline effect was clearly dependent on choice of assay (Fig. 3, *panels E* and *F*). Metabolic activity assessed by MTT appeared to be paradoxically increased (~20%, $P \leq 0.04$) across most concentrations of ergovaline with a 24-h treatment period (*panel E*), whereas alamarBlue indicated an equivalent decrease in metabolic activity ($P \leq 0.007$). Deoxyribonucleic acid remained unchanged, indicating a lack of cell loss after 24-h treatment. Extending the treatment period to 72 h (*panel F*) resulted in an ~10% loss in DNA, reflecting cell loss with most concentrations of ergovaline ($P \leq 0.07$). The same decrease in metabolic activity (alamarBlue and MTT) was seen only with 10^{-4} *M* ergovaline (14%, $P \leq 0.0001$ and 12%, $P \leq 0.006$, respectively). The increase in MTT seen with some ergovaline concentrations after 24 h on days 11 and 21 was repeated on day 21 after 72 h with 10^{-9} to 10^{-5} *M* ($P \leq 0.02$).

All assay results indicate that undifferentiated Caco-2 cells are more sensitive to ergovaline than differentiated cells. Although the concentration required for extreme toxicity is relatively high (10^{-4} *M*), this concentration was included in the range of ergovaline tested on primary cultures of rat pituitary cells (10^{-4} to 10^{-8} *M*, Strickland et al., 1994) and primary cultures of bovine dorsal pedal vein endothelial cells (Table 2, Strickland et al., pers. comm.).

The basis for this differential sensitivity may relate to the cell's capacity for antioxidant activity. Duthie and Collins (1997) measured catalase and glutathione reductase (GR) in Caco-2 cells from days 0 to 10 in culture and found activity of both enzymes to increase with time. Numerous reports indicate that the antioxidant enzyme activity of these cells also increases as d in culture after confluence increase (Baker and Baker, 1992; Cepinskas et al., 1994; Bestwick and Milne, 1999; Karczewski and Nordhoek, 1999). These enzymes include superoxide dismutase (SOD), catalase, GR, and glutathione S-transferase. The increase in these enzymes reported by Bestwick and Milne (1999) was concomitant with a decrease in reactive oxygen species (ROS).

Literature has documented the presence of antioxidant enzymes in GI tissue. Grisham et al. (1990) reported that normal human colon epithelial cells had catalase, SOD, and glutathione (GSH) peroxidase activities that were 5-, 2-, and 1.3-fold those measured in Caco-2 cells harvested at confluence. Liver enzyme activity was 12-fold higher for catalase and fourfold higher for SOD and GSH peroxidase compared with colon epithelial cell activity. Superoxide dismutase and GSH peroxidase were also found in piglet colon and ileum (Crissinger et al., 1989). Activity of these two enzymes was age dependent, yet negatively correlated to each other, i.e., from day 1 to 30 GSH peroxidase increased in both ileum and colon samples, whereas SOD decreased. Based on additional data, these authors concluded that the neonatal piglet intestine had a lower capacity for dealing with the presence of hydrogen peroxide than that of an older animal.

Vasoconstriction has long been known to be one of the effects of ergot alkaloids and is also associated with ischemia. Enhanced antioxidant activity would be required to deal with increasing ROS. The cell culture system used herein contains no vascular component, therefore toxicity is not being mediated via the vasculature. Instead, ergovaline must be exerting its toxic effect directly on the GI cells. The ramifications of these findings are important when one considers that our exposure period is relatively brief (72 h) compared with the exposure period of an animal feeding on endophyte-

contaminated pasture (containing ergovaline). Although differentiated cells may be comparatively resistant to ergovaline, the toxic effect on dividing cells would be reflected in a lack of intestinal integrity because undifferentiated cells fail to divide and replace sloughed differentiated cells. Such a scenario may be the explanation for the enteritis of the small intestine of pigs fed ergot alkaloids for 9 wk (Whittemore et al., 1977).

Another indication of a direct cellular effect of ergot alkaloids, independent of vasculature, involves bovine liver mitochondrial activity. Both ergonovine and ergotamine were found to inhibit bovine liver mitochondrial adenosine triphosphatase (ATPase) activity (ergonovine is a derivative of the lysergic acid moiety found in ergovaline, with the carboxylic acid at C-8 replaced by amide linkage to $-CHCH_3CH_2OH$; ergotamine is an ergovaline derivative having one methyl group on the peptide moiety replaced by benzyl group; Moubarak et al. [1998]). These findings may help to explain the paradoxical increase in MTT activity found when differentiated cells were treated with ergovaline. Although the chromophore used in the alamarBlue assay is an electron acceptor that replaces molecular oxygen at the end of the electron transport chain, the MTT chromophore is dependent on succinate dehydrogenase activity, upstream in the tricarboxylic acid (TCA) cycle, for its reduction. If ATPase activity is inhibited, a compensatory elevation in TCA cycle activity might be expected. If inhibition of ATPase activity resulted in decreased electron transport chain activity, this would explain findings reported here (Fig. 3, *panels C, E,* and *F*) that indicate concomitant decreases in alamarBlue with increasing MTT values.

Data from others have also found this increased MTT in response to ergot alkaloids. Table 2 shows increased MTT reduction by bovine dorsal pedal vein endothelial cells when exposed to various ergot alkaloids (Strickland et al., pers. comm.). Although most compounds were toxic at 10^{-4} *M* (24-h exposure), several compounds had increased MTT values at lower concentrations (i.e., ergovaline 10^{-6} *M*, α-ergocryptine 10^{-5} *M*, loline 10^{-5} *M*, and *N*-acetyl loline 10^{-8} *M*). Perhaps, the variable MTT response across ergot alkaloid concentrations in vascular endothelial cells is related to the response reported here for the Caco-2 cells. Both a toxic and "stimulatory or mitogenic" effects of ergovaline and other ergot alkaloids were also found when bovine vascular smooth muscle cells were exposed in vitro (Strickland et al., 1996). Again, as assessed by MTT, in quiescent cultures, cells appeared to grow with 10^{-9} *M* ergovaline, whereas 10^{-6} and 10^{-9} *M* ergovaline inhibited growth in growing cultures. It is the author's supposition that ergot alkaloid treatments may be causing alterations in the quantity of enzyme, enzyme activity, or possibly substrate availability, and thereby disrupting the "control state" ratio of reduced MTT to cell number. In the case of ratio disruption, the MTT assay becomes invalid for use as an indicator of cell number.

Conclusions

As found in bovine vascular smooth muscle cells (Strickland et al., 1996), ergovaline was toxic to dividing Caco-2 cells. Differentiated Caco-2 cells were much less sensitive to ergovaline. The highest concentrations for which toxicity was found could be considered pharmacological. This cell culture system has the advantage that differentiation state–dependent toxicity can be explored, which is impossible in an in vivo experiment. Unfortunately, longer exposure periods (that would more typically reflect in vivo field conditions) using undifferentiated cells are not possible with this system. The toxicity observed in vitro may be indicative of physiological effects and may play an important role in the etiology of fescue toxicosis. These findings delineate nontoxic conditions for future ergovaline transport studies.

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